SYNTHESIS OF DESOXYRIBONUCLEIC ACID IN LETHAL AMPHIBIAN HYBRIDS [1]

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It has been found that by cross-fertilization between certain combinations of related amphibian species lethal hybrids arise (Baltzer, 1934; Brachet, 1944; Moore, 1946). These lethal hybrids stop developing at a stage corresponding morphologically to early gastrula. Microscopical examination has shown that various abnormalities occur in the hybrid embryos, *e.g.* irregular mitoses and pycnosis (Schönmann, 1938; Brachet, 1944). When the controls have reached stage 13 (early neurula) all the cells, except those in the blastoporal region, have died (Schönmann, 1938; Moore, 1948).

The results of Hoff-Jørgensen and Zeuthen (1952; *cf.* also Løvtrup, 1954) have disclosed that no increase in total DNA occurs during the early development, up to a late blastula stage. This can only mean that the DNA used in the formation of new nuclei during the early stages of development must be present as a reserve in the mature oocyte. Hoff-Jørgensen and Zeuthen could demonstrate that the size of this store corresponds to the DNA content in about 2700 diploid nuclei in *Rana platyrrhinus*, and that it, to a major extent, is localized in the cytoplasm. It is a quite obvious suggestion that the developmental block observed in hybrids may reflect the lack of ability to synthesize substances necessary for continued development. On the basis of the observations mentioned above it would seem possible that DNA might be one of these substances. In that case one might expect the hybrid embryos to develop until the DNA reserves were exhausted. Although there is a discrepancy as to the morphological stage at which the DNA exhaustion and the developmental block occur, it was still considered worth while to test this possibility.

A necessary condition for doing this work was that the DNA analyses were carried out by the microbiological assay of DNA (desoxyribosides) worked out by Hoff-Jørgensen (1951), as the other available methods of DNA determination seem to be too unspecific or too insensitive to estimate DNA when present in such low concentrations as in early amphibian embryos (*cf.* the discussion by Løvtrup, 1954).

MATERIAL AND METHODS

Embryos

Normal *Rana pipiens* embryos, and *Rana pipiens* ♀ × *Rana sylvatica* ♂ hybrid embryos obtained by stripping *R. pipiens* eggs directly from the same female

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donors of control eggs into suspensions of *R. sylvatica* sperm, were reared in 10% Ringer's solution, without bicarbonate or phosphate, at a temperature of 15° C.

Preparation of dry samples

A single tube of dry material was prepared as follows. Jelly was removed with forceps from 25 embryos, which were then pipetted into a 10 × 75 mm. test tube. Excess medium was removed, and the tube nearly filled with acetone. The embryos were then crushed thoroughly with a ball-tipped glass rod. The tube was then centrifuged, the supernatant decanted, the precipitate re-suspended in acetone, re-centrifuged, decanted again, and left in a desiccator over calcium chloride for 24 hours. The open end of the tube was then drawn out and sealed off, and the tube stored until analyses for DNA could be made.

Hybrid and control embryos were prepared simultaneously. Each tube was labelled with the developmental stage number (Shumway, 1940) of the controls and with a designation of the time (in hours) elapsing since fertilization.

Determination of DNA [3]

The principle of the microbiological DNA assay is that a certain strain of lactic acid bacteria, *Thermobacterium acidophilus* R 26, is unable to grow in the absence of desoxyribosides. The extent of growth is almost proportional to the concentration of these substances in the culture medium. By measuring the growth obtained after adding an aliquot of the biological material to a culture medium free of desoxyribosides, it is possible to estimate the content of DNA. Before the microbiological assay is carried out the DNA in the samples is first hydrolyzed by the following procedure: To each tube of dry material is added 0.5 ml. of 0.5 N NaOH, and the tube placed in a boiling water bath for 15 minutes. After this is added 2.5 ml. buffer-activator solution (0.06 *M* maleic acid; 0.01 *M* $MgSO_4$), and the pH is checked and adjusted to 6.5–7.0 if necessary. Then 0.1 mg. crystalline desoxyribonuclease, contained in 0.1 ml. is added, and the tubes incubated 20 hours at 37° C. After this the sample is transferred to a volumetric flask, the volume made up to 5 ml., and DNA assayed as described by Hoff-Jørgensen (1951).

It may be argued that this method is not specific for DNA, as it determines the sum of DNA and free desoxyribosides. It should be noticed, however, that determinations on extracts not treated with desoxyribonuclease generally give negligible values (*cf.* the discussion following the paper by Hoff-Jørgensen, 1954). This observation thus leads to the not surprising conclusion that free desoxyribonucleosides are rarely found in the cells, even in embryos. It seems reasonable, therefore, to apply the name "DNA" to the substances determined by the present method, namely, those which liberate desoxyribonucleosides upon treatment with desoxyribonuclease. This does not imply anything with respect to the degree of polymerization of the substances, about which nothing is known. However, there is certainly no reason to believe that it is present in a high-polymeric form, ready for incorporation in the nuclei arising during segmentation. From a genetical point of view it would rather seem required that of the DNA incorporated during

[3] All the determinations of DNA were carried out in the laboratory of Dr. E. Hoff-Jørgensen. We gladly acknowledge our gratitude for this assistance.

the early development at least 50 per cent, *viz.* the DNA in the male chromosomes, be synthesized from rather low-molecular substances. The constancy of DNA during segmentation should not, therefore, be taken to demonstrate that no DNA synthesis occurs. Rather it indicates that DNA is synthesized at the expense of preformed desoxyribosides, and that no *total* synthesis of DNA occurs.

Results

The results are shown in Figure 1. The DNA content at the beginning of development is 0.063 ± 0.002 μg (standard deviation: ± 0.005 μg). This value is calculated as the average of five determinations (only three of these are shown

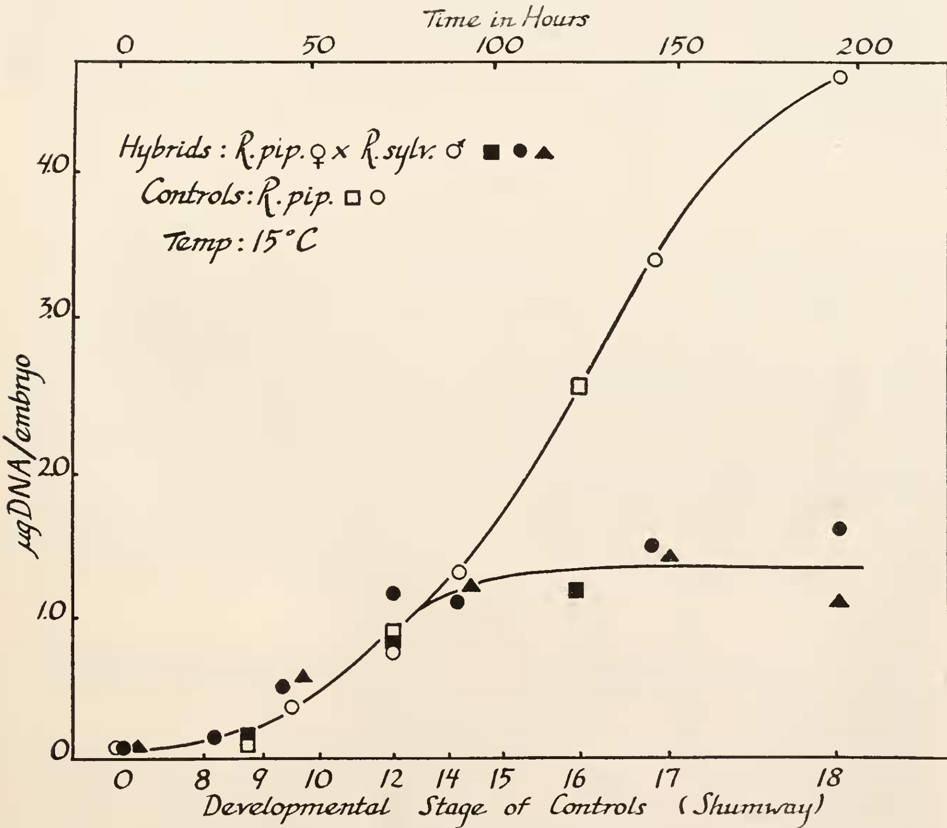

FIGURE 1. Desoxyribonucleic acid content of normal and hybrid embryos at various stages of development.

in the figure) on eggs from 2 to 4 hours after fertilization (2–8 cells). The content is the same in hybrids (0.065; 0.065) and in controls (0.068; 0.059; 0.058). According to Sze (1953) the DNA content of a diploid nucleus in *R. pipiens* is 10.4×10^{-6} μg. The reserve would thus correspond to about 6000 nuclei.

At the end of segmentation the content of DNA begins to rise in both hybrids and controls, the rate of synthesis increasing gradually for about 50 hours, *i.e.*, until the controls have reached stage 14 (Shumway). At this time the synthesis of DNA ceases in the hybrids, the final content calculated as the average of the last five values being 1.31 ± 0.08 μg DNA (standard deviation ± 0.17 μg). In the controls synthesis continues at constant rate (linear increase) until between stages 17 and 18, when a decrease in rate of synthesis is observed. There are rather few points on the control curve, but the shape of the curve is exactly the same as found in *R. platyrrhinus* (Hoff-Jørgensen and Zeuthen, 1952; Løvtrup, 1954). It should also be mentioned that the absolute amounts are approximately the same in the two species; thus the content in the oocyte of the European species is about 0.065 μg according to the former authors. As the DNA content per nucleus is almost twice as high in *R. platyrrhinus* nuclei, the reserve supply corresponds to that of only 2700 nuclei. The highest value found in *R. pipiens* is 4.62 μg at stage 18, and in *R. platyrrhinus* a value close to 5 μg was found (Hoff-Jørgensen and Zeuthen, 1952; Løvtrup, 1954).

Discussion

It is seen right away that the possibility mentioned in the introduction has been disproved by the results, *i.e.*, the hybrid embryos are able to synthesize DNA, and do so at normal speed until the early neurula stages have been reached.

The results obtained thus clearly demonstrate a dissociation between DNA synthesis and morphological development. The next question to consider is whether there is a dissociation between DNA synthesis and cell division (formation of new nuclei). During development the DNA of the hybrids is increased about 20-fold, corresponding to 120,000 cells. Were cell division lagging behind DNA synthesis to any appreciable extent, an accumulation of DNA would occur. No indication of such accumulation has been observed (*cf.* Schönmann, 1938; Brachet, 1952), but this unfortunately cannot be considered decisive evidence, as DNA may not always be detectable by cytochemical methods. Thus the DNA reserve in the amphibian oocyte cannot be demonstrated by the Feulgen reaction (*cf.* Brachet, 1952, and the discussion in Løvtrup, 1954).

The decrease in cell size which takes place during early amphibian development seems, according to Schönmann, to occur also in the hybrids. Without settling this question definitely, this observation seems to suggest that cell division proceeds normally, in spite of the abnormal mitoses.

It has been observed that the animal (blastoporal) region survives longer than other parts of the hybrid embryos (Baltzer, 1934). It has been shown by transplantation that both inductive power and capacity for further differentiation are not lost (Lüthi, 1938; Brachet, 1944; Moore, 1947, 1948). As the DNA synthesis proceeds normally in the hybrids until the early neurula stages, it would seem reasonable to suggest that this synthesis corresponds to cell divisions localized in the blastoporal (animal) region. The major part of the cells formed here will in the normal embryo invaginate and form the mesoderm. If this interpretation is correct, it seems possible from our results to divide the pre-larval DNA synthesis (cell division) into two phases, the first of which (mesodermal) is found in both hybrids and controls. The initiation of the second (linear) phase ap-

parently is conditioned by a normal gastrulation. It would seem that this second (ectodermal) phase to some extent is associated with the development of the neural tissues, epidermis, etc. It is interesting in this connection to note that a third phase of DNA synthesis, correlated at least partly with the development of the endoderm, has been found to begin during early larval development (*cf.* Løvtrup, 1954).

Much work has already been devoted to the study of the physiology and biochemistry of hybrid development. Thus the results of Steinert (1951) indicate that RNA also is synthesized in hybrid embryos, although to a lesser extent than in the controls. This finding is in agreement with the fact that very little RNA is synthesized before neurulation. In normal embryos a considerable increase in respiratory rate occurs during gastrulation. Contrary to this, only a very slight increase has been found in the hybrids (Barth, 1946; Chen, 1952). In agreement with this is the observation that carbohydrate is consumed at a very low rate in the hybrid embryos (Gregg, 1948).

It is difficult to correlate these various findings, but it seems warranted at least to conclude that only a minor fraction of the total energy consumed during gastrulation is used for DNA synthesis and cell division.

Summary

1. The DNA contents of normal *R. pipiens* embryos and *R. pipiens* ♀ × *R. sylvatica* ♂ embryos at various developmental stages have been estimated by the method of Hoff-Jørgensen (1951).

2. The DNA content of *R. pipiens* embryos remains constant from fertilization until near the end of segmentation, at which point it increases at a constant rate until shortly before muscular movements begin.

3. The DNA content of hybrid embryos (which do not gastrulate) is identical with that of *R. pipiens* embryos until the latter reach the neural fold stage, at which point it does not increase further.

4. The significance of these results is discussed briefly.

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